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GUINEA-PIG TEST OF THE VIRULENCE OF DIPH- THERIA BACILLI.*†

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No method for testing the virulence of diphtheria bacilli is ideal, because it is impossible to conduct a practical test under the same conditions which the bacilli encounter on gaining entrance into the human body. Probably no one practical method can be considered absolutely reliable. The object is to secure the most favorable conditions for the production of toxin, and to test it on a suitable animal which does not possess any marked degree of natural immunity. Toward this end certain steps in technic have been advanced from time to time as favorable to a reliable test, and it is only by taking advantage of each step that one can secure a technic as satisfactory as we can expect in the present light of our imperfect knowledge of the formation of diphtheria toxins. While investigators report their cultures of diphtheria bacilli as virulent or non-virulent, as the case may be, the majority do not give the technic whereby such conclusions were reached, and certainly the technic has much influence on the result. Consequently quite a number of workers have a hazy idea of this subject. It is the object of our paper briefly to review the more important tests with which we are familiar and to give one which has given us uniform satisfaction.

As a practical measure the guinea-pig test is used under the following conditions:

1. Cases harboring bacilli morphologically similar to the diphtheria bacillus over a long period of time. These cases include those who have recovered from clinical diphtheria, and contact cases. Quarantine is often inconvenient and irksome, both to the patient and the attending physician, especially when it is prolonged. Cases of clinical diphtheria often harbor virulent bacilli three and four weeks and even longer after the disappearance of every symp-

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tom, when the patient is healthy in every other respect. A test demonstrating this fact helps to pacify the patient and to convince him of the wisdom of measures tending to prevent the spread of infection. Again, persons after contact with cases of clinical diphtheria frequently harbor virulent bacilli, which are just as dangerous as the bacilli from the patient. On the other hand, some persons may harbor perfectly harmless non-virulent bacilli. In both these instances local treatment is of little avail in getting rid of the bacilli. It is impossible by microscopic examination alone to claim that any culture does or does not possess virulence. We know now that certain types are often found non-virulent, but in our experience we never have been able to foretell the result with confidence by microscopic examination alone. Nor can we recognize with certainty the pseudo-diphtheria bacillus, whether the term be applied to non-virulent diphtheria bacilli or to the organism known as Hofmann's bacillus. Since it never has been definitely proven that a non-virulent organism can become virulent, and since the weight of evidence is against such a view, we must not depend on microscopic examination which tells us no more than that bacilli morphologically similar to the diphtheria bacillus are present, but on a reliable animal test for the virulence of the bacilli.

2. Frequently the discharges from infected ears show a diphtheria-like bacillus. Such cases usually pursue a prolonged course and the question of virulence is a serious one, especially when the patient seeks admission into a hospital, school, etc. We have isolated the bacilli from over 100 such cases following scarlet fever in order to prepare autogenous vaccines; 57 per cent gave a diphtheria-like bacillus—in pure culture, 35 per cent, and in combination with another organism, 22 per cent. Twenty-two of these were tested for virulence with negative results. On the other hand, several of our most potent toxins were obtained from bacilli isolated from ear discharges in the diphtheria department. These cases of ear infection especially require the aid of test on guinea-pigs for proper management.

3. One should always be cautious in giving a positive diagnosis in cases of nasal infection, because the Hofmann's bacillus is frequently present. We have often found solid types of bacilli re-

sembling this bacillus to be virulent, and therefore we never feel justified in giving a negative report by microscopic examination alone and trusting that the bacilli are not virulent.

Any technic to be of value as a practical routine measure should give (1) a reliable and decisive result in the shortest time possible; (2) it should be the least expensive possible. At best the test on guinea-pigs is a costly one, and this factor is of much importance where the demand for tests is likely to be large.

In order to secure a strong toxin representative of the bacilli tested it is necessary to observe the following points: (1) The broth used for making the toxin should be distinctly alkaline to litmus, at least "0.5 per cent acid to phenolphthalein"; (2) it should contain a sugar, as 1 per cent glucose; (3) the culture should be grown at 37° C. for at least 48 hours. We believe that enough soluble toxins are generated in this time to make a reliable test, although there is no doubt but that in a large number of cases, stronger toxin results after seven days' growth. If the test can be made in a leisurely manner and especially if it is desired to obtain a strong toxin, as in the manufacture of antitoxin, it is well to secure a good surface growth by "educating" the bacilli and to test the product between the seventh and ninth day.

A crude test can be made by injecting an emulsion made by mixing a loopful of bacilli with sterile normal saline solution. The main objection to this method is the fact that the dose cannot be well regulated. If the pig dies, the assumption is that death was due to diphtheria, and when injecting a second pig with toxin-antitoxin mixture, it is desirable to have some control of the dosage. In case the pig survived, it would be necessary to make a toxin and try that. In this way two animals would be used.

There is a difference of opinion concerning the value of filtered and unfiltered cultures. We have frequently seen the unfiltered culture kill a pig when the filtrate did not, both being made in exactly the same manner and given in the same dose. Antitoxin protected a second pig, each time showing that we were dealing with diphtheria toxin. Escherich¹ never obtained a negative result when using the filtrate in doses corresponding to 0.5 per cent

¹ Lehmann and Neumann, *Med. Hand Atlas*, Saunders & Co., 1901, p. 399.

of the body weight of the pig. In his virulent cultures, about 0.05 per cent sufficed. We believe in the use of unfiltered cultures after giving both methods extensive trial, for as Theobald Smith¹ has written: "We may, for convenience, regard the disease-producing power of diphtheria bacilli as made up of two elements: toxicity and virulence. The former represents the rate of accumulation of toxin in culture fluids, and is easily measured; the virulence on the other hand, which may be regarded as the behavior of diphtheria bacilli toward living tissue, is as yet an unknown quantity." If we use the filtered toxin, "but an instrument of pathological power is here dealt with, and under artificial conditions, since we do not know the nature of the nutritive fluid which the bacteria make use of on mucous membrane, nor as a consequence, whether the toxin production in bouillon is a true index of the production of toxin in mucous membrane."

We have employed the following technic in the Philadelphia Hospital for Contagious Diseases. We wish to state here, that it has been worked out in its essentials according to the technic of Rosenau,² in establishing the legal immunity unit for standardizing diphtheria antitoxin.

1. If under a microscope the bacillus has the morphology of *B. diphtheriae*, it is restained by Gram's method. If it is Gram negative (that is, does not retain the stain after having been washed with alcohol), we conclude that it is not a diphtheria bacillus, for no diphtheria bacillus has ever been found Gram negative. Should it be Gram positive, the organism is isolated.

2. Isolation is readily accomplished by "streaking" on a Petri dish of blood serum, or by inoculating the water of condensation in large tubes of gelatin-agar and passing the water once over the surface of the medium. Usually within 24 hours the colonies are well developed and can be picked out. The bacilli are stained again with methylene-blue and Gram to ascertain their purity and morphological characteristics.

3. When a pure culture is obtained it is planted in slightly alkaline (0.5 per cent to phenolphthalein) broth containing 1 per

¹ Nuttall and Graham, *Bacteriology of Diphtheria*, Cambridge University Press, 1908, p. 173.

² *Bull. No. 21*, Hyg. Lab., U.S. Public Health and Marine Hospital Service, Washington, pp. 35-72.

cent glucose and grown for 48 hours at 37.5° C. The tubes are slanted in order to expose as large a surface as possible to the oxygen in the tube. During this time soluble toxins are generated in sufficient quantities to kill a guinea-pig, supposing of course the bacillus to be a virulent diphtheria bacillus.

4. A healthy guinea-pig is selected, weighing about 250 and not over 300 gms. Rosenau states that "in general terms the guinea-pig as a vital factor in the standardization of antitoxin is quite as dependable as our ordinary weights and measures." The dose, 0.5 per cent of the body weight of the pig expressed in cubic centimeters, is accurately measured and poured into the barrel of the syringe. Thus a 250-gm. pig would receive 1.25 c.c. of (unfiltered) culture. We use and warmly recommend the syringe devised by Dr. Hitchens.¹ The syringe is thoroughly washed with absolute alcohol followed by sterile salt solution, and the tip of the needle plugged with sterile vaselin. In the side arm of the syringe enough sterile salt solution is poured to bring the total quantity of injection up to 4 c.c. An assistant holds the pig; the abdomen is dampened with alcohol and then sterile salt solution "and the needle introduced in the left flank from one-half to an inch above the supraspinous process of the ileum. The needle is introduced its whole length (1.25 inch), strictly subcutaneously toward the middle line. This part of the operation is arranged so that the point of the needle will be just about at the linea alba." The culture is then injected, the syringe turned in order that the salt solution may flow out of the side arm down the barrel of the syringe, and this is injected without removing either the needle or the bulb. It is very necessary to avoid entering the peritoneum or piercing the skin too superficially, and special care must be observed to avoid injecting the toxin into muscles, as emphasized by Meltzner and Auer.² By following these directions, several conditions are assured: (a) "The rate of absorption is always the same; (b) the pressure effects are always the same; (c) the relation of toxin to body juices is constant when the injection is made always in the same anatomical structures"; (d) by using percentage of body

¹ *Jour. Exp. Med.*, 1906, 8, p. 647.

² *Am. Med.*, 1905, 5, p. 75.

weight of culture to weight of animal, the dose is always the same, regardless of the size of the animal.

5. The animal is kept under daily observation four to five days. Symptoms of toxemia are usually manifest in 36 hours, in which case the pig is inclined to nestle in some corner, the hair bristled, appetite poor, etc. He usually dies in four to six days, or may recover. He may be sick for a day or so and recover, and later show signs of paralysis. If at the end of four to six days the animal is apparently healthy, the organism is said to be non-virulent. The late paralytic effects of toxone appear about the second to third week. If the animal dies within four to six days an autopsy is performed in order to establish the cause of death; the possibility of death from other infections, as pneumonia, tuberculosis, etc., must not be left out of consideration. The condition of the site of inoculation, of the spleen, the suprarenals, the pleurae, the heart, etc., is carefully observed. Smears and cultures are made from the site of inoculation, the pleural and peritoneal cavities, heart's blood, and spleen. If diphtheria-like bacilli are recovered, it is justifiable to conclude that they killed the animal; but it is not justifiable to conclude that they are diphtheria bacilli. To be sure of this, it is necessary to go a step further and inject a second pig protected with antitoxin.

6. A new sub-culture is made in the same manner and from the same source as the one used for the first test; the same dose is injected plus 250 units of antitoxin. The culture and antitoxin are measured out and mixed, and allowed to stand at room temperature for an hour before injection. This gives ample time for the toxins to be neutralized by the antitoxin. The remaining antitoxin immunizes the animal against toxins formed in the body by the living bacteria injected. The question of the amount of antitoxin needs no discussion. One unit neutralizes 100 times the least fatal dose of toxin, so that 250 units would immunize against 25,000 fatal doses, or more than 25 c.c. of virulent toxins. It is rarely necessary to inject more than 2.5 c.c. of culture, so that there still remains enough antitoxin to immunize against 11 times the amount injected. This is the final test. If the second pig lives, it is proof sufficient that the organisms were virulent diph-

theria bacilli. If it dies, they were not, and although they killed both pigs, they cannot produce diphtheria.

It may be well to add here a few words in reference to toxones. The identity of this substance is still unsettled, although Erlich's views are widely accepted. He believes that toxones constitute definite secretory products of the diphtheria bacillus, but differ from toxin in having a weaker affinity for antitoxin and in the toxophore group. Toxones do not produce death even in large doses, but are believed to cause the paralyses which frequently develop about the second or third week of the disease. We have seen paralyses develop in the guinea-pig at the end of the first week. Should a patient harboring diphtheria-like bacilli capable of producing paralyses be given a clean bill of health? In practically all cases if a second pig is protected with antitoxin, no paralyses occur. So far the assumption has been that only the toxins are capable of producing clinical diphtheria, and if the culture is free of toxic action, the organisms are considered sufficiently harmless to give the patient his freedom. Personally we have always regarded such strains with suspicion, for it seems that if the bacillus produces sufficient toxone to give paralysis, it is likely to be in some degree a toxin producer, although we have seen guinea-pigs recover after being slightly sick for a few hours or a day and yet be seized with typical paralyses about two weeks after the injection. The subject of toxones is still quite unsettled.

The following is a list of tests and results that have been made in the course of routine work in the Philadelphia Hospital for Contagious Diseases from February 15, 1910, to October 15, 1910.

Number of tests in which filtered toxin was given.....	25
Result Positive.....	2
Result Negative.....	23
Number of tests in which unfiltered culture was given.....	67
Result Positive.....	10
Result Negative.....	57

Filtered and unfiltered cultures from the same case were given two separate pigs in 8 cases.